Non-Radioisotopic Kit for Measuring Protein kinase C Activity

CycLex PKC Super Family Kinase Assay Kit

Cat# CY-1175

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Intended Use

The CycLex Research Product **CycLex PKC Super Family Kinase Assay Kit** is primarily designed to measure the activities of purified Protein kinase C (PKC) for the rapid and sensitive evaluation of inhibitors or activators. The phospho-specific monoclonal antibody used in this assay kit has been demonstrated to recognize the phospho-threonine38 residue in CPI-17, which is efficiently phosphorylated by PKC. Additionally, column fractions of cultured primary cell, cell line, or tissue homogenate can be assayed for PKC activity with the CycLex Research Product **CycLex PKC Super Family Kinase Assay Kit** if the appropriate dose of Protein kinase C specific inhibitor e.g. H-9 is used.

Applications of this kit include:

1) Monitoring the purification of Protein kinase C.
2) Screening inhibitors or activators of Protein kinase C
3) Detecting the effects of pharmacological agents on Protein kinase C activity.

This assay kit is for research use only and not for use in diagnostic or therapeutic procedures.

Storage

- Upon receipt store all components at 4°C.
- Don't expose reagents to excessive light.

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Introduction

Members of the protein kinase C (PKC) family of serine/threonine protein kinases have been implicated in numerous cellular responses in a large variety of cell types. PKC isoenzymes are involved in multiple biochemical processes relevant to cell growth, differentiation, and transformation, and play critical roles in transducing signals from a plethora of extracellular receptors, including those for hormones, neurotransmitters, growth factors, and antigens (1, 2).

At present, the PKC family of serine/threonine-specific protein kinases includes eleven known members that are expressed in a variety of tissues and cell types. Based on similarity in primary amino acid sequence and domain structure, the distinct PKC isoenzymes are grouped into three subfamilies. Members of the Ca^{2+}-dependent subfamily are termed conventional, or cPKC, and include PKCα, the two alternatively spliced forms of the β gene, PKC βI and βII, and PKC γ (3-6). These isoenzymes contain three conserved domains, namely, the diacylglycerol-binding C1 domain, which contains two repeats of a cysteine-rich zinc finger-like domain, the phospholipid- and Ca^{2+}-binding C2 domain, and the catalytic C3/C4 domains. Members of the second subfamily, termed novel, or nPKC, are Ca^{2+}-independent for their activity and include PKC δ, ε, η, θ and μ (7-10). The C2-like N-terminal domain of these isoenzymes can bind acidic phospholipids but not Ca^{2+} ions. A third PKC subfamily termed atypical, or aPKC, includes only two members, PKCζ and ι/λ, that possess a single cysteine-rich domain which cannot bind phospholipids or phorbol esters (7, 11, 12).

Regulation of PKC activity is mediated by defined cofactors that interact with specific regions in its regulatory domain (13) as well as transphosphorylation by serine/threonine kinases (14) and autophosphorylation (15). The activation is accompanied by removal of the basic pseudosubstrate region from the kinase domain (16) and may involve the association with specific proteins, termed receptors for activated PKC (17), that were suggested to function as selective scaffolds for activated PKC at discrete subcellular compartments.

Distinct PKC isoenzymes exhibit differences in tissue distribution, intracellular localization, and cofactor requirements, suggesting that they are independently regulated through response to discrete ligands, and that they may possess distinct protein substrates (18). It is possible that the specific combination of isoenzymes expressed in a given cell determines the outcome of the PKC-dependent response in that particular cell.

Measurement of Protein kinase C activity

The protocol generally regarded as most sensitive for the quantitative measurement of Protein kinase C activity involves incubation of the Protein kinase C sample with substrate, either a natural or synthetic polypeptide (such as a histone III-S), in the presence of $Mg^{2+}$and ^{32}P-labeled ATP. The reaction is terminated by "spotting" a sample onto a phosphocellulose P81 filter paper disc, followed by washing extensively to remove unincorporated radiolabel and the incorporated radioactivity on P81 filter is counted. While sensitive, this method is labor-intensive, generates hazardous radioactive waste, and depends on a radioisotope of short half-life. It is particularly unsuitable when kinase assays are only performed on an infrequent basis. The CycLex Research Product **CycLex PKC Super Family Kinase Assay Kit** uses a peroxidase coupled anti-sequence-specific phosphoserine monoclonal antibody as a reporter molecule in a 96-well ELISA format. This assay provides a non-isotopic, sensitive and specific method to detect Protein kinase C activity.

The CycLex Research Product **CycLex PKC Super Family Kinase Assay Kit** is designed to accurately determine the presence and relative amount of Protein kinase C activity in purification column fractions, and for the non-isotopic kinetic analysis of Protein kinase C activity. Careful attention to extraction methods and the assay protocol will provide the investigator with a reliable tool for the evaluation of Protein kinase C.

The **CycLex PKC Super Family Kinase Assay Kit** can be used for measuring most of recombinant

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PCK isozymes including all cPKCs as well as aPKCs and several nPKC, e.g. PKC δ, ε and θ (see page 18).

Principle of the Assay

The CycLex Research Product **CycLex PKC Super Family Kinase Assay Kit** is a single-site, semi-quantitative immunoassay for Protein kinase C activity. Plates are pre-coated with a substrate corresponding to recombinant CPI-17 (Protein kinase C-potentiated inhibitor protein of 17kDa), which contains threonine38 residues that can be efficiently phosphorylated by Protein kinase C. The detector antibody is AK-1F11, an antibody that specifically detects only the phosphorylated CPI-17 threonine38. The CycLex Research Product **CycLex PKC Super Family Kinase Assay Kit** might be used to determine the presence of Protein kinase C activity in purification column fractions, or to follow the kinetics of a purified Protein kinase C as well as screening Protein kinase C inhibitor or activator. To perform the test, the sample is diluted in Kinase Buffer, pipetted into the wells and allowed to phosphorylate the bound substrate in the presence of Mg^{2+} and ATP. The amount of phosphorylated substrate is measured by binding it with a AK-1F11, a anti-phospho-CPI-17 threonine38 monoclonal antibody, followed by binding with horseradish peroxidase conjugated anti-mouse IgG, which then catalyzes the conversion of the chromogenic substrate tetra-methylbenzidine (TMB) from a colorless solution to a blue solution (or yellow after the addition of stopping reagent). The color is quantified by spectrophotometry and reflects the relative amount of Protein kinase C activity in the sample. For kinetic analysis, the sample containing Protein kinase C is added to the wells in a similar fashion and at varying times the reaction is stopped by the addition of a chelator, sodium ethylenediaminetetraacetate (EDTA) and the amount of phosphorylated substrate determined as before.

The CycLex Research Product **CycLex PKC Super Family Kinase Assay Kit** is designed to determine non-isotopic kinetic analysis of Protein kinase C. Careful attention to extraction and purification methods and the assay protocol will provide the investigator with a reliable tool for the evaluation of Protein kinase C activity.

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Summary of Procedure

Add 100 µL of sample to the wells

↓ Incubate for 30 min at 30°C

Wash the wells

↓

Add 100 µL of Anti-phospho-CPI-17 Threonine38 monoclonal antibody (ST-3B11).

↓ Incubate for 30 min at room temp.

Wash the wells

↓

Add 100 µL of HRP conjugated anti-mouse IgG

↓ Incubate for 30 min at room temp.

Wash the wells

↓

Add 100 µL of Substrate Reagent

↓

Add 100 µL of Stop Solution

↓

Measure absorbance at 450 nm

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Materials Provided

All samples and standards should be assayed in duplicate. The following components are supplied and are sufficient for the one 96-well microtiter plate kit.

Microplate: One microplate supplied ready to use, with 96 wells (12 strips of 8-wells) in a foil, zip-lock bag with a desiccant pack. Wells are coated with recombinant CPI-17 as a substrate.

10X Wash Buffer: One 100 mL bottle of 10X buffer containing 2%Tween®-20

Kinase Buffer: One bottle containing 20 mL of 1X buffer; used for Kinase Reaction Buffer and sample dilution.

10X Phosphatidylserine (PS): One vial containing 1.2 mL of 500 µg/ml Phosphatidylserine.

50X $CaCl_2$: One vial containing 0.4 mL of 125 mM $CaCl_2$, used for Kinase Reaction Buffer (Ca/PS plus)

50X EGTA: One vial containing 0.4 mL of 100 mM EGTA, used for Kinase Reaction Buffer (Ca/PS minus)

20X ATP: Lyophilized ATP Na_2 salt.

Anti-Phospho-CPI-17-T38 Monoclonal Antibody (AK-1F11): One vial containing 12 mL of anti-phospho-CPI-17-T38 monoclonal antibody (AK-1F11). Ready to use.

HRP conjugated Anti-mouse IgG: One vial containing 12 mL of HRP (horseradish peroxidase) conjugated anti-mouse IgG. Ready to use.

Substrate Reagent: 20 mL of the chromogenic substrate, tetra-methylbenzidine (TMB). Ready to use.

Stop Solution: One bottle supplied ready to use, containing 20 mL of 1 N H_2SO_4. Ready to use.

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Materials Required but not Provided

- **Protein kinase C from rat brain:** Available from Sigma (Cat # P0329); Positive control should be added to the first well at around 10 m units/well. (Unused Protein kinase C enzyme should be stored in aliquots at below -70°C.)

- **10X H-1 (100 µM):** KN-62 is available from Sigma, Cat#. A-7795. 10 mM stock solution (DMSO) diluted 1:100 in Kinase Buffer.

- **Pipettors:** 2-20 µL, 20-200 µL and 200-1000 µL precision pipettors with disposable tips.

- **Precision repeating pipettor**

- **Wash bottle or multichannel dispenser** for plate washing.

- **Microcentrifuge and tubes** for sample preparation.

- **Plate reader** capable of measuring absorbance in 96-well plates at dual wavelengths of 450 nm/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. The plate can also be read at a single wavelength of 450 nm, which will give a somewhat higher reading.

- **500 or 1000 mL graduated cylinder**

- **Reagent reservoirs**

- **Deionized water of the highest quality**

- **Disposable paper towels**

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Precautions and Recommendations

- Store the protein kinase C at below -70°C and the ATP at -20°C in aliquots. Store all other components at 4°C. Do not expose reagents to excessive light. Avoid freeze/thaw cycles.
- Allow all the components to come to room temperature before use.
- All microplate strips that are not immediately required should be returned to the zip-lock pouch, which must be carefully resealed to avoid moisture absorption.
- Do not use kit components beyond the indicated kit expiration date.
- Use only the microtiter wells provided with the kit.
- Rinse all detergent residue from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- The buffers and reagents used in this kit contain Kathon-CG as preservatives. Care should be taken to avoid direct contact with these reagents.
- Do not mouth pipette or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Dispose of tetra-methylbenzidine (TMB) containing solutions in compliance with local regulations.
- Avoid contact with Substrate Solution which contains hydrogen peroxide.
- Avoid contact with Stop Solution which contains Sulfuric Acid.
- In case of contact with the Stop Solution and the Substrate Solution, wash skin thoroughly with water and seek medical attention, when necessary.
- **Biological samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.**
- **CAUTION: Sulfuric Acid is a strong acid. Wear disposable gloves and eye protection when handling Stop Solution.**

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Detailed Protocol

The CycLex Research Product **CycLex PKC Super Family Kinase Assay Kit** is provided with removable strips of wells so the assay can be carried out on separate occasions using only the number of strips required for the particular determination. Since experimental conditions may vary, an aliquot of the protein kinase C from rat brain (Cat # P0329), available from Sigma, should be included in each assay as a positive control. Disposable pipette tips and reagent troughs should be used for all liquid transfers to avoid cross-contamination of reagents or samples.

Preparation of Working Solution

1. Prepare a working solution of **Wash Buffer** by adding 100 mL of the **10X Wash Buffer** (provided) to 900 mL of ddH_2O. Mix well. Store at 4°C for two weeks or -20°C for long-term storage.

2. Prepare **20X ATP Solution** by adding **1.6 mL** of ddH_2O to the vial of 20X ATP (provided, lyophilized). Mix gently until dissolved. the Final concentration of the **20X ATP Solution** should be **1.25 mM**. Store the solution in small aliquots (e.g. 100 µL) at -20°C.

3. Agitate **10X Phosphatidylserine (PS)** vigorously by sonication or Vortex for a minute prior to use on ice.

4. Prepare **Kinase Reaction Buffer (Ca/PS plus)** by mixing following reagents.

	96 assays	10 assays	1 assay
Kinase Buffer (provided)	**8.3 mL**	**830 µL**	**83 µL**
20X ATP (provided)	**0.5 mL**	**50 µL**	**5 µL**
50X $CaCl_2$ (provided)	**0.2 mL**	**20 µL**	**2 µL**
10X PS (provided)	**1.0 mL**	**100 µL**	**10 µL**
Total	**10 mL**	**1000 µL**	**100 µL**

You will need 80-90 µL of Kinase Reaction Buffer (Ca/PS plus) per assay well. Mix well. Discard any unused Kinase Reaction Buffer (Ca/PS plus) after use.

5. Prepare **Kinase Reaction Buffer (Ca/PS minus)** by mixing following reagents.

	96 assays	10 assays	1 assay
Kinase Buffer (provided)	**8.3 mL**	**830 µL**	**83 µL**
20X ATP (provided)	**0.5 mL**	**50 µL**	**5 µL**
50X EGTA (provided)	**0.2 mL**	**20 µL**	**2 µL**
H_2O	**1.0 mL**	**100 µL**	**10 µL**
Total	**10 mL**	**1000 µL**	**100 µL**

In the case of assaying individual column fractions, we recommend you to measure the kinase activity in the absence of Calcium/PS as well as in the presence of these in parallel.

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Standard Assay

1. Remove the appropriate number of microtiter wells from the foil pouch and place them into the well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C.

2. Prepare all samples (diluted with Kinase Buffer as needed). All samples should be assayed in duplicate.

3. To assay individual column fractions, add **10 µL of each fraction** to the wells of the assay plate on ice. Duplicate wells containing 10 m units/10 µL protein kinase C (Sigma Cat # P0329) should be included in each assay as a positive control for phosphorylation.

4. Begin the kinase reaction by addition of **90 µL Kinase Reaction Buffer (Ca/PS plus)** and/or **Kinase Reaction Buffer (Ca/PS minus)** per well, cover with plate sealer, and incubate **at 30°C for 30 minutes**.

5. Wash wells five times with Wash Buffer making sure each well is filled completely. Remove residual Wash Buffer by gentle tapping or aspiration.

6. Pipette **100 µL** of **Anti-Phospho-CPI-17-T38 Monoclonal Antibody AK-1F11** into each well, cover with plate sealer or lid, and incubate **at room temperature (ca.25°C) for 30 minutes**. Discard any unused antibody after use.

7. Wash wells five times as same as in step 5.

8. Pipette **100 µL** of **HRP-conjugated Anti-mouse IgG** into each well, cover with plate sealer or lid, and incubate **at room temperature (ca.25°C) for 30 minutes**. Discard any unused conjugate after use.

9. Wash wells five times as same as in step 5.

10. Add **100 µL** of **Substrate Reagent** to each well and incubate **at room temperature (ca.25°C) for 5–15 minutes**.

11. Add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.

12. Measure absorbance in each well using a spectrophotometric plate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the plate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution.

Note-1: Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

Note-2: Reliable signals are obtained when either O.D. values do not exceed 0.25 units for the blank (no enzyme control), or 2.5 units for the protein kinase C positive control.

Note-3: If the microplate reader is not capable of reading absorbance greater than the absorbance of the Wee1 positive control, perform a second reading at 405 nm. A new O.D. values, measured at 405 nm, is used to determine protein kinase C activity of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

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Kinetic Assay

1. Remove the appropriate number of microtiter wells from the foil pouch and place them into the well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C.

2. Prepare all enzyme samples (diluted with Kinase Buffer as needed). All enzyme samples should be assayed in duplicate.

3. To assay enzyme sample, add **10 µL** of **each enzyme sample** or **protein kinase C** (Cat # P0329) to the wells of the assay plate. Duplicate wells containing 10 m units/10 µL protein kinase C (Sigma Cat # P0329) should be included in each assay as a positive control for phosphorylation.

4. Begin kinase reaction by addition of **90 µL Kinase Reaction Buffer (Ca/PS plus)** in duplicate per well in timed intervals (suggested interval is 1 minutes but should be individually determined for each system). After the final addition, incubate **at 30°C for 20 minutes**.

5. Stop the reaction by flicking out the contents. (Alternatively, the reaction may be terminated by the addition of 150 µL 0.1 M Na EDTA, pH 8.0 to each well).

6. Wash wells five times with Wash Buffer making sure each well is filled completely. Remove residual Wash Buffer by gentle tapping or aspiration.

7. Pipette **100 µL** of **Anti-Phospho-CPI-17-T38 Monoclonal Antibody AK-1F11** into each well, cover with plate sealer or lid, and incubate **at room temperature (ca.25°C) for 30 minutes**. Discard any unused antibody after use.

8. Wash wells five times as same as in step 6.

9. Pipette **100 µL** of **HRP-conjugated Anti-mouse IgG** into each well, cover with plate sealer or lid, and incubate **at room temperature (ca.25°C) for 30 minutes**. Discard any unused conjugate after use.

10. Wash wells five times as same as in step 6.

11. Add **100 µL** of **Substrate Reagent** to each well and incubate **at room temperature (ca.25°C) for 5-15 minutes**.

12. Add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.

13. Measure absorbance in each well using a spectrophotometric plate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the plate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution.

Recommendations

Special considerations when screening activators and inhibitors

In order to estimate the inhibitory effect on Protein kinase C activity in the test chemicals correctly, it is necessary to conduct the control experiment of "Solvent control" at least once for every experiment

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and "Inhibitor control" at least once for the first experiment, in addition to "Test sample", as indicated in the following table. When test chemicals cause an inhibitory effect on Protein kinase C activity, the level of A450 is weakened as compared with "Solvent control". The high level of A450 is not observed in "Inhibitor control" (usually A450<0.2).

Assay reagents	Test sample	Solvent control	Inhibitor control
Kinase Reaction Buffer (Ca/PS plus)*	**80 µL**	**80 µL**	**80 µL**
10X Inhibitor or equivalent	**10 µL**	**-**	**-**
Solvent for Inhibitor	**-**	**10 µL**	**-**
10X H-9 (100 µM)**	**-**	**-**	**10 µL**
Protein kinase C (1 m unit/µL)* or your enzyme sample**	**10 µL**	**10 µL**	**10 µL**

* Kinase Reaction Buffer (Ca/PS plus): See page 8, section "Preparation of Working Solution"
** 10X H-9: See page 6, section "Materials Required but not Provided"
*** Sigma Cat # P0329: See page 6, section "Materials Required but not Provided"

1. Following the above table, add the Reagents to each well of the microplate. Finally, initiate reaction by adding 10 µL of Protein kinase C to each well and mixing thoroughly at room temperature. Cover with plate sealer. Incubate **at 30°C for 30 minutes**.

2. Follow the **Standard Assay**, steps 5-12, page 9.

Special considerations when measuring precise Protein kinase C activity

In order to measure the activity of Protein kinase C correctly, it is necessary to conduct the control experiment of "Inhibitor control" at least once for every experiment and "Ca/PS minus control" at least once for the first experiment, in addition to "No enzyme control" as indicated in the following table. Although the level of A450 increases in "Test sample" when Protein kinase C enzyme activity is in the sample, the high level of A450 is not observed in "Inhibitor control", "ATP minus control" and "No enzyme control".

Assay reagents	Test Sample	Inhibitor control	Ca/PS minus control	Positive control	No enzyme control
Kinase Reaction Buffer (Ca/PS plus)*	**80 µL**	**80 µL**	**-**	**80 µL**	**80 µL**
Kinase Reaction Buffer (Ca/PS minus)*	**-**	**-**	**80 µL**	**-**	**-**
10X H-9 (100 µM)**	**-**	**10 µL**	**-**	**-**	**-**
Kinase Buffer	**10 µL**	**-**	**10 µL**	**10 µL**	**10 µL**
Buffer for Your enzyme fraction	**-**	**-**	**-**	**-**	**10 µL**
Protein kinase C (1 m units/µL)***	**-**	**-**	**-**	**10 µL**	**-**
Your enzyme fraction	**10 µL**	**10 µL**	**10 µL**	**-**	**-**

* Kinase Reaction Buffer (Ca/PS plus) and (Ca/PS minus): See page 8, section "Preparation of Working Solution"
** 10X H-9: See page 6, section "Materials Required but not Provided"
*** Sigma Cat # P0329: See page 6, section "Materials Required but not Provided"

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1. Following the above table, add the Reagents to each well of the microplate. Finally, initiate the reaction by adding 10 µL of "Your enzyme fraction" or "Buffer" to each well and mixing thoroughly at room temperature. Cover with plate sealer. Incubate **at 30°C for 30 minutes**.

2. Follow the **Standard Assay**, steps 5-12, page 9.

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Evaluation of Results

1. Average the absorbance values for the Protein kinase C sample duplicates (positive control) and all experimental samples duplicate values (when applicable). When the Protein kinase C (10 m units/assay) is included as an internal control for the phosphorylation reaction, the absorbance value should be greater than 1.0 with a background less than 0.2.

2. For screening of purification/chromatography fractions, on graph paper, plot the mean absorbance values for each of the samples on the Y-axis versus the fraction number on the X-axis to determine the location of the eluted, purified Protein kinase C.

3. For kinetic analysis, on graph paper, plot the mean absorbance values for each of the time points on the Y-axis versus the time of each reaction (minutes) on the X-axis.

Assay Characteristics

The CycLex Research Product **CycLex PKC Super Family Kinase Assay Kit** has been shown to detect the Protein kinase C activity in column fractions of human or animal cell lysates. The assay shows good linearity of sample response. The assay may be used to follow the purification of Protein kinase C.

It should be noted that this assay kit detects not only Protein kinase C activity but also other protein kinase activities, e.g. Protein kinase A (activated by partial proteolysis), Rho-kinase and ILK, in crude extract and column sample in the absence of Ca^{2+} and phospholipid.

Troubleshooting

1. The Protein kinase C positive control should be run in duplicate, using the protocol described in the **Detailed Protocol**. Incubation times or temperatures significantly different from those specified may give erroneous results.

2. The reaction curve is nearly a straight line if the kinetics of the assay is of the first order. Variations in the protocol can lead to non-linearity of the curve, as can assay kinetics that are other than first order. For a non-linear curve, point to point or quadratic curve fit methods should be used.

3. Poor duplicates, accompanied by elevated values for wells containing no sample, indicate insufficient washing. If all instructions in the **Detailed Protocol** were followed accurately, such results indicate a need for washer maintenance.

4. Overall low signal may indicate that desiccation of the plate has occurred between the final wash and addition of Substrate Reagent. Do not allow the plate to dry out. Add Substrate Reagent immediately after wash.

Reagent Stability

All of the reagents included in the CycLex Research Product **CycLex PKC Super Family Kinase Assay Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, kit reagents should be stored at 4°C, except the ATP must be stored at -20°C. Coated assay plates should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack.

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Sample Preparation

Numerous extraction and purification methods can be used to isolate Protein kinase C. The following protocols have been shown to work with rat cerebellum as enzyme sources, and are provided as examples of suitable methods. Concentrated or highly purified protein kinase C should be diluted. It is strongly advised that the user always perform an initial experiment to determine the proper dilution to be used in subsequent experiments. This need not be any more than a single time point assay using serial dilutions of the crude extract, cell lysate or sample fraction taken prior to a purification step. One eight well strip of the substrate plate should be sufficient for this initial experiment. All sample preparation should be performed at 4°C and recovered fractions should be kept at 4°C to prevent loss of enzymatic activity.

CAUSION: It should be noted that this assay kit detects not only Protein kinase C activity but also other protein kinase activities, e.g. Protein kinase A (activated by partial proteolysis), Rho-kinase and ILK, in crude extract and column sample in the absence of Ca^{2+} and phospholipid. You should trace Protein kinase C protein level by western blotting in column fractions.

Preparation of rat brain extract

1. Homogenize fresh 10-15 g of rat brain in four volumes of ice-cold Extraction Buffer (20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 5 mM EDTA, 5 mM EGTA, 20 mM β-mercaptoethanol, 1 mM PMSF, 1 μg/mL pepstatin, 0.5 μg/mL leupeptin, 5 mM β-glycerophosphate, 2 mM NaF, 2 mM Na_3VO_4) in a Potter-Elvehjem tissue homogenizer.

2. Centrifuge the homogenate for 20 min. at 20,000 x g to pellet the insoluble membrane/organelle fraction.

3. The supernatant was centrifuged at 100,000 X g for 1 hr.

Column Purification of conventional Protein kinase C

4. Load the resultant high-speed supernatant onto a 2 x 8 cm column of DEAE-Sephacel (Amersham Biosciences) or equivalent anion-exchange resin, equilibrated with Buffer A (20 mM Tris-HC1 buffer, pH 7.5, containing 1 mM EGTA, 1 mM EDTA, 10 mM β-mercaptoethanol, 0.5 mM PMSF, 1 μg/mL pepstatin, 0.5 μg/mL leupeptin, 5 mM β-glycerophosphate, 2 mM NaF and 2 mM Na_3VO_4)

5. Wash the column with six column volumes of Buffer A.

6. Sequentially elute the protein with a 0-0.3 M KC1 gradient in Buffer A. Pool the fractions containing high kinase activity and concentrate by ultrafiltration to 1/5 original volume and adjust the KC1 concentration to 1.5 M.

7. Load the DEAE eluate (kinase rich fraction) to a phenyl-Sepharose column (1 X 5 cm), equilibrated with Buffer B (20 mM Tris-HC1 buffer, pH 7.5, containing 0.5 mM EGTA, 0.5 mM EDTA, 10 mM β-mercaptoethanol, 0.5 mM PMSF, 1 μg/mL pepstatin, 0.5 μg/mL leupeptin, 5 mM β-glycerophosphate, 2 mM NaF and 2 mM Na_3VO_4 and 10 % glycerol) containing 1.5 M KCl.

8. Wash the column with Buffer B containing 1.5 M KCl until the absorbance at 280 nm reached baseline.

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9. Elute protein kinase C with a gradient of 1.5-0 M KC1 in Buffer B. Pool the fractions containing high kinase activity and concentrate by ultrafiltration to approximately 2 ml.

10. Dialyzed the pooled kinase fraction against Buffer C (0.02 M KPO_4, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT and 5 mM β-glycerophosphate, 2 mM NaF and 2 mM Na_3VO_45 and 10 % glycerol) for 2 hr at 4°C.

10. Load the phenyl-Sepharose column eluate onto a hydroxylapatite column (2 ml) equilibrated Buffer C.

11. Washed the column with buffer C until the absorbance at 280 nm reached baseline.
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12. Elute protein kinase C with a gradient of 0.02-0.28 M KPO_4 in Buffer C at 0.5 ml/min, collecting 1 ml fractions.

Preparation of cell lysate

1. Grow cells on 10 cm dish at 80 % confluent overnight.

2. Treat the cells with desired drug or compound for appropriate time.

3. Scrape the cells with a rubber policeman after wash the cells three times with cold PBS.

4. Resuspend the cell pellet with 500 µL of an appropriate extraction buffer (for example; 20 mM Tris-HCl, pH 7.5, 75 mM NaCl, 5 % glycerol, 0.01 % Triton-X100, 20 mM β-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 1 µg/mL pepstatin, 0.5 µg/mL leupeptin, 5 mM β-glycerophosphate, 2 mM NaF, 1 mM Na_3VO_4).

5. Sonicate the resuspended cells on ice for 30-60 seconds, using 10-15 second pulses.

6. Centrifuge at 100,000 x g for 1 hour at 4°C.

7. Aliquot cleared cell lysate to a clean microfuge tube. These samples are now ready for analysis according to the instructions provided in the **Detailed Protocol**. Store the cell lysate at below -70°C.

NOTE: THE ABOVE PROCEDURES ARE INTENDED ONLY AS A GUIDELINE. THE OPTIMAL EXPERIMENTAL CONDITIONS WILL VARY DEPENDING ON THE PARAMETERS BEING INVESTIGATED, AND MUST BE DETERMINED BY THE INDIVIDUAL USER. NO WARRANTY OR GUARANTEE OF PERFORMANCE USING THESE PROCEDURES IS MADE OR IMPLIED.

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Example of Test Results

Fig.1 Dose dependency of Protein kinase C enzyme reaction

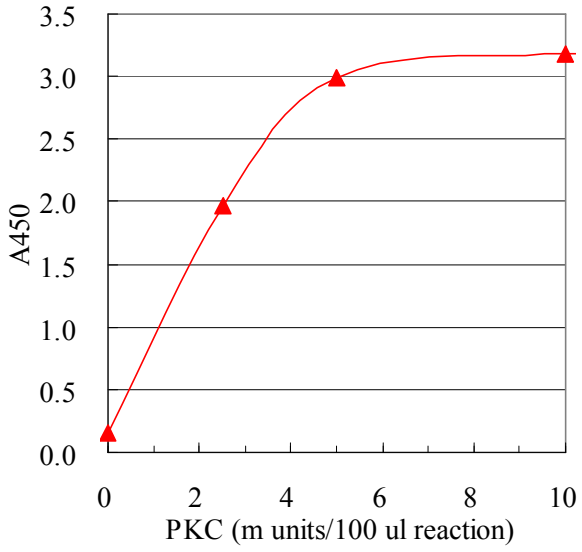

Fig.2 Time course of rat brain Protein kinase C enzyme reaction

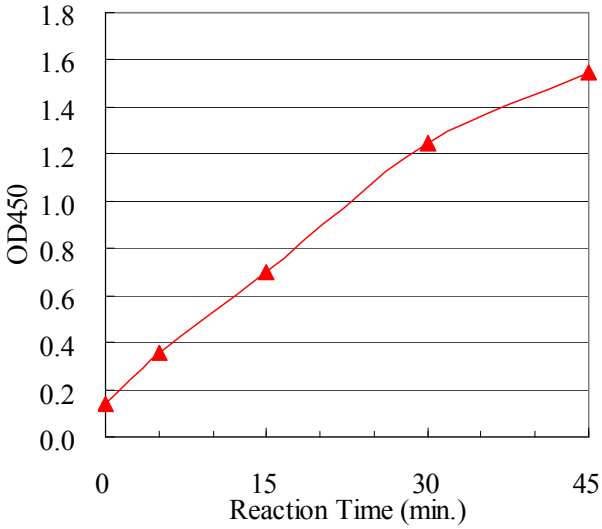

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Fig.3 Effect of specific protein kinase C inhibitor H-9 on activity of rat brain Protein kinase

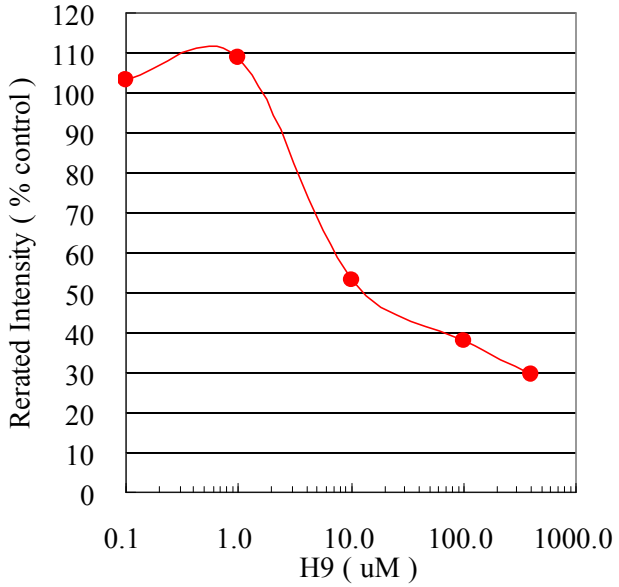

Fig.4 RESOURCE Q column elution profile of Protein kinase C from rabbit brain extract

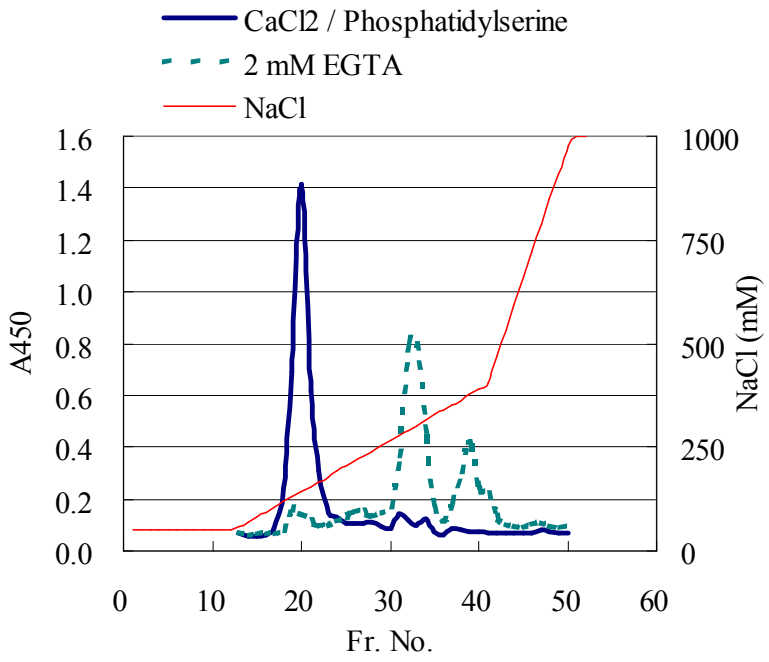

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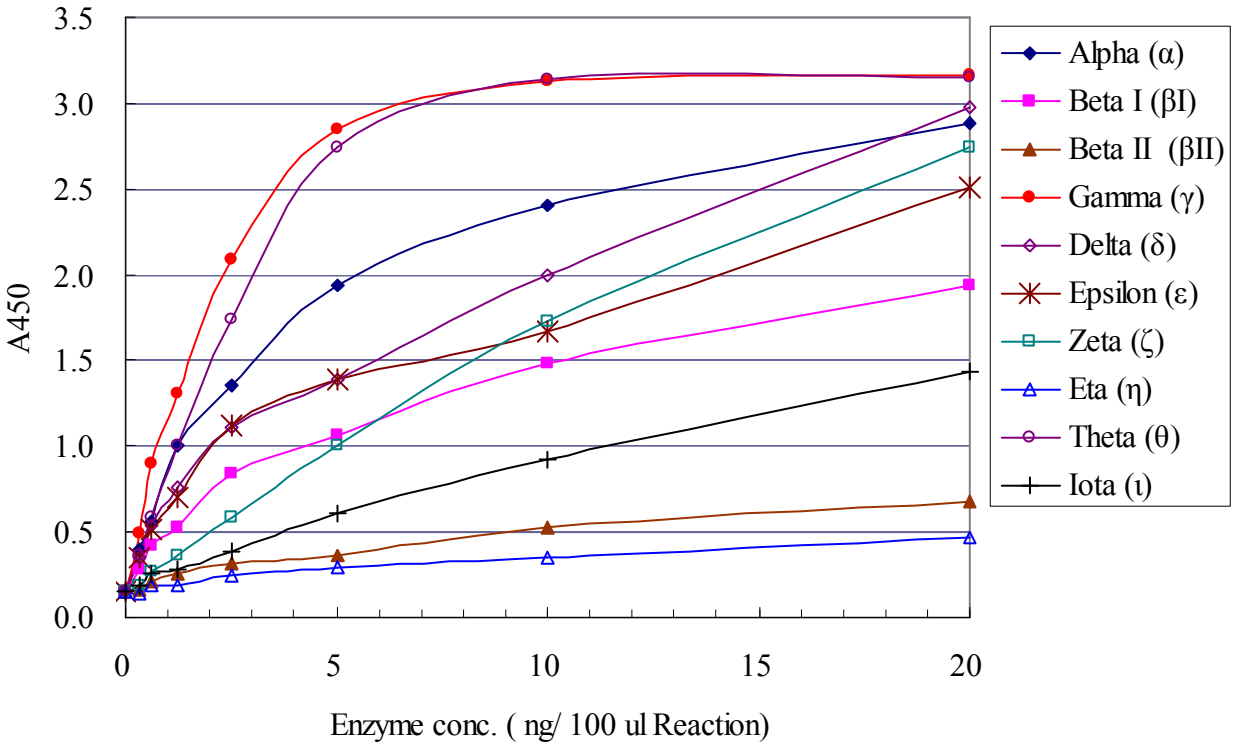
Fig.5 Detectable activities of protein kinase C isozymes using CycLex PKC Super Family Kinase Assay Kit

cPKC	
PKC α	++++
PKC βI	+++
PKC βII	+
PKC γ	+++++
nPKC	
PKC δ	++++
PKC ε	++++
PKC η	+
PKC θ	+++++
PKC μ	N/A
aPKC	
PKC ζ	++++
PKC ι / λ	++



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*** Anti-Phospho-CPI-17 Thr38 Monoclonal Antibody (Clone AK-1F11): Cat# CY-M1024**

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